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Germination of *Rhizopus oligosporus* Sporangiospores

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The morphology of *Rhizopus oligosporus* (NRRL 2710) sporangiospores and their physiological requirements for germination were studied. Germination proceeded in two separable phases: phase I (swelling) and phase II (germ tube protrusion). The optimal conditions for germination were 42°C and pH 4.0. Sporangiospores contained insufficient endogenous carbon for swelling or germination to occur in distilled water. Initial swelling during phase I occurred only in the presence of a suitable carbohydrate. Subsequent production of germ tubes during phase II required exogenous sources of both carbon and nitrogen. Spores germinated most rapidly in mixtures of amino acids; L-proline and L-alanine were the most effective. These amino acids, at concentrations as low as 10^{-6} M, supported germination when combined with glucose and McIlvaine (citric acid-phosphate) buffer. D-Glucose, D-xylose, and D-mannose were the most effective carbohydrates tested for promotion of germination.

Tempeh, a fermented soyfood long used as a meat substitute in Indonesia (6), is prepared by inoculating cooked soybeans, or soy-grain mixtures, with *Rhizopus oligosporus* sporangiospores. The fermentation, which usually occurs at 30 to 32°C, requires ca. 30 h to complete. Mycelial growth becomes visible after ca. 20 h. The initial stage of the fermentation involves germination and outgrowth of the sporangiospore inoculum.

Hesseltine et al. (3) and Sorensen and Hesseltine (5) have studied the physiology of *R. oligosporus* mycelial growth. The germination requirements of its sporangiospores, however, are not well established. Knowledge of optimal germination conditions for sporangiospore starters may allow tempeh manufacturers to accelerate the critical initial period of the fermentation, thus minimizing the possibility of subsequent overgrowth by contaminants. Some physical and nutritional conditions promoting germination of *R. oligosporus* sporangiospores and the accompanying morphological changes that occur during their outgrowth are presented in this paper.

MATERIALS AND METHODS

Organism. *R. oligosporus* NRRL 2710 was obtained from the U.S. Department of Agriculture Northern Regional Research Laboratory (Peoria, Ill.), courtesy of C. W. Hesseltine. This strain has been used extensively for the production of soybean tempeh in the United States.

Media. Protein digests (NZ amine type A, soy peptone, and Hy-Cas) were obtained from Sheffield Chemical Co., Norwich, N.Y.

A modification of the defined medium described by Graham et al. (2) was used for sporangiospore production. A base medium consisting of 0.75% glucose, 0.09% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, and 2.4% Ionagar no. 2S (Wilson Diagnostics, Glenwood, Ill.) was prepared in distilled water. After autoclaving, the base medium was aseptically combined with an equal volume of sterile McIlvaine buffer (pH 4.0). The mixture was held at 50°C in a water bath. Stock solutions of filter-sterilized thiamine hydrochloride (25 μg/ml) and trace metals (0.2 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $ZnSO_4 \cdot 7H_2O$, and 0.2 mg of $MnSO_2 \cdot 4H_2O$ per ml) were aseptically added to the medium, using 0.1 ml per 100 ml of medium. The medium was dispensed into standard-size plastic petri dishes. Various formulations of this medium in liquid form were utilized for shake-flask studies.

Sporangiospore production and preservation. Sporangiospores were produced on the medium described above, using a plate-overlay method. A base layer of the defined medium (15.0 ml) was overlaid with 5.0 ml of 1.2% Ionagar containing ca. 10^5 sporangiospores per ml. Plates were incubated at 30°C for 5 days. Sporangiospores were harvested with a wire loop, using two consecutive rinsings (10.0 ml each) of sterile physiological saline solution containing 0.01% Tween 80.

Sporangiospore suspensions were preserved on activated silica gel by a modification of the method described by Perkins (4). Tubes of silica gel were prepared by weighing 5.0 g of chromatography-grade silica gel (0.05 to 0.2 mm; 70-325 mesh ASTM) (E. Merck AG, Darmstadt, Germany) into 50-ml screw-cap test tubes. The capped tubes were then oven-sterilized at 180°C overnight. Sterile tubes of anhydrous silica gel were placed in an ice bath. A 1.0-ml portion of spore suspension was pipetted along the surface of the silica gel, dropwise. Tubes were returned to the ice bath for 15 min. Each tube was mixed with a tube agitator and sealed with a layer of Parafilm. The spore suspensions were stored at 4°C. Estimates of spore viability were determined by using Mycophil agar (BBL Microbiology Systems, Cockeysville, Md.). Viability of the spore preparations ranged from 60 to 65% over the course of these experiments.

Assessment of germination. For these experiments, germination was defined as the extension of a germ tube to a length equal to one-half the diameter of the spore. Germination was reported as a percentage of the spore population, determined by microscopic count. Germination in both liquid and solid media was evaluated.

Germination in liquid media was determined by using 50-ml shake flasks (Bellco Glass, Inc., Vineland, N.J.) containing 10.0 ml of medium. Flasks were inoculated with ca. 5×10^6 sporangiospores and incubated in an environmental incubator-shaker (model G24; New Brunswick Scientific Co., Inc., Edison, N.J.) set at 150 rpm. Samples were removed from the flasks with sterile 100-μl capillary pipettes. The percentage of germinated spores was determined microscopically with a hemacytometer. At least 400 spores were counted for each sample.

Germination was also evaluated on a solid agar medium

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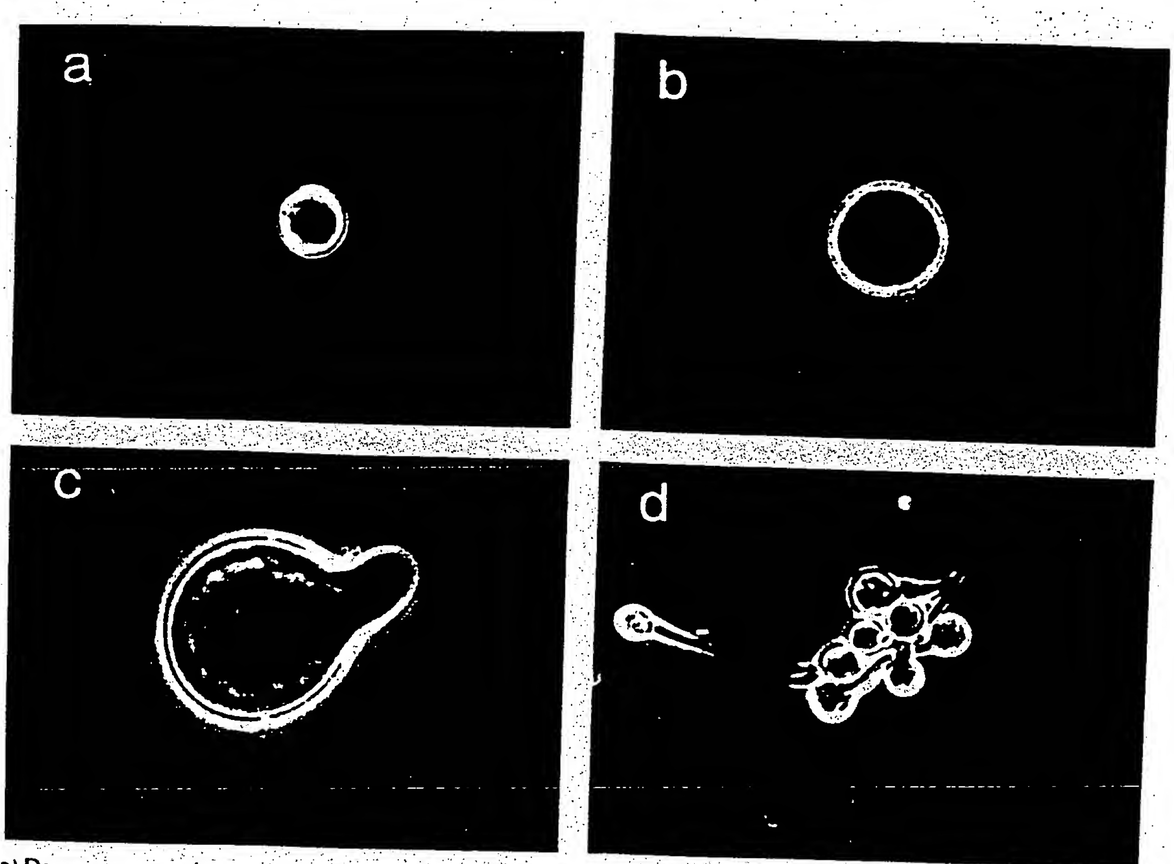

FIG. 1. (a) Dormant sporangiospore. Magnification, ×1,000. Diameter, 5.1 μm. (b) Sporangiospore after 4 h of incubation (42°C) in defined medium. Magnification, ×1,000 (phase-contrast). Diameter, 12 μm. (c) Germinating sporangiospore after 6 h of incubation (42°C) in defined medium. Magnification, ×1,000 (phase-contrast). (d) Germinated sporangiospores forming mycelial pellets after 6.5 h in defined medium. Magnification, ×150 (phase-contrast).

(6.0 ml per plate) in standard-size petri dishes. A sporangiospore inoculum (ca. 5×10^6 spores) was spread over the surface of each plate with an alcohol-flamed glass rod. After incubation for 2 to 8 h, agar blocks were aseptically removed with a scalpel. The blocks were placed on cleaned microscope slides under cover slips and examined under 450× magnification. For each sample, at least 400 spores were counted. Microscopic fields were selected randomly.

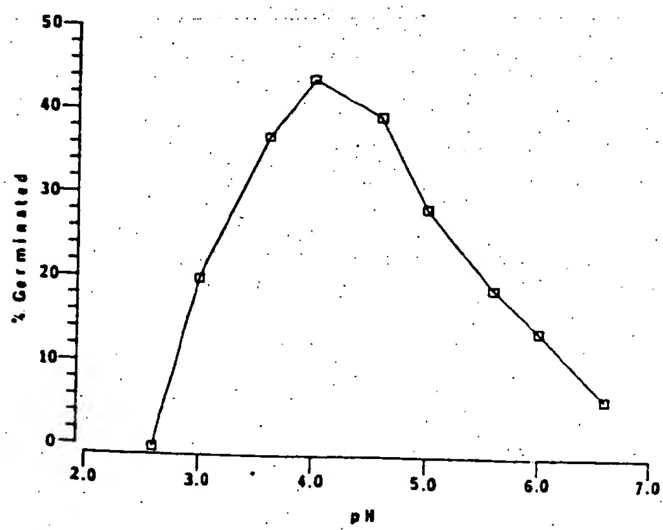

FIG. 2. Effect of pH on germination of *R. oligosporus* sporangiospores. Spores were incubated in the defined medium (42°C) and scored at 5 h.

RESULTS

Germination sequence of *R. oligosporus* sporangiospores. The germination sequence of *R. oligosporus* sporangiospores is shown in Fig. 1a to c. Germination proceeded through two distinguishable phases. Phase I involved the enlargement of the spore (Fig. 1b) before the emergence of a

TABLE 1. Requirements for swelling and germination of *R. oligosporus* sporangiospores[a]

Medium	Condition of spores after 24 h at 42°C[b]	
	Spore diam (μm)	Germination (%)
Distilled water	5.5	0
McIlvaine (citric acid-Na_2HPO) buffer (pH 4.0)	5.5	0
Buffer–0.37% glucose	8.3	0
Buffer–0.05% $(NH_4)_2SO_4$	5.6	0[c]
Buffer–0.37% glucose–0.05% $(NH_4)_2SO_4$	12.1	60

[a] Viability of the spore suspension (based on Mycophil agar plate count) was 65%.

[b] Mean, based on 50 observations.

[c] 7% of the spores germinated at 30°C and formed chlamydospores in this medium.

germ tube (phase II) (Fig. 1c). Swelling of the spores during phase I varied with the medium. In the defined liquid medium, sporangiospores swelled to an average diameter of 12.1 µm, from 5.1 µm, before the emergence of a germ tube (Fig. 1c). Germlings tended to clump together during extended incubation periods to form mycelial pellets (Fig. 1d). Because of this phenomenon, which interfered with germination determinations, solid media were used for experiments requiring incubation periods longer than 6 h.

Influence of pH and temperature on germination. The effect of pH on germination of sporangiospores (Fig. 2) was determined during a 6-h incubation (42°C) in the defined medium. The buffer in the base medium was prepared according to the specified pH values. The optimal pH range for germination was 3.6 to 4.6; maximum germination was at pH 4.0. Germination was delayed and reduced to 15 to 20% after 18 h at pH values below 2.6 or above 6.6.

The effect of incubation temperature on sporangiospore germination was determined by using the defined medium at pH 4.0. Germination was scored after incubation for 6 h at 26, 30, 34, 38, 42, 46, or 50°C. Germination was most rapid at 42°C. After extended incubation (18 to 24 h), germination (ca. 25%) was also observed at 26 and 46°C.

Nutritional requirements for germination. Sporangiospores were incubated in media lacking one or more constituents of the defined medium (Table 1). Sporangiospores incubated f r 24 h in either distilled water or buffer did not swell or germinate. Spores incubated in buffer with 0.37% glucose did not germinate after 24 h; however, after 6 h of incubation, ca. 60% of the spores swelled to an average diameter of 8.3 µm. Swollen spores underwent a loss in refractility and became granular in appearance. Approximately 60% of the sporangiospores incubated in a medium containing carbon and nitrogen sources but no trace elements in a buffer base

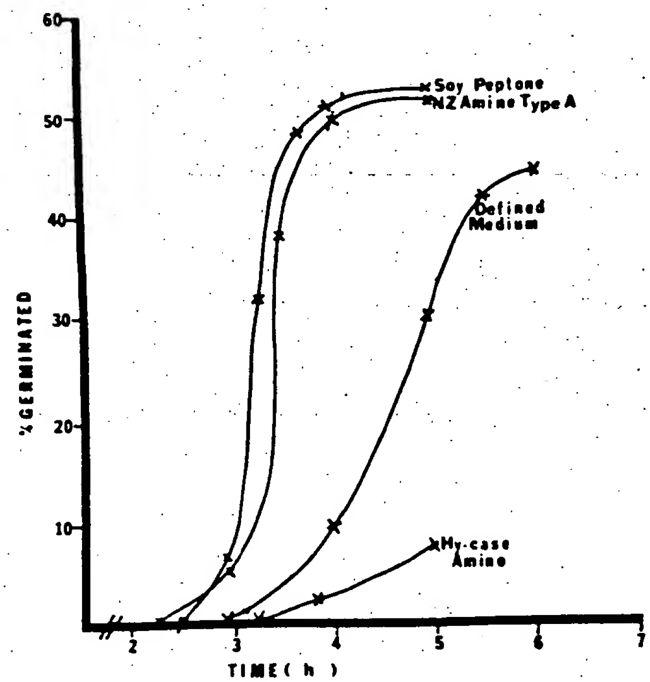

FIG. 3. Comparison of germination kinetics in defined and complex media. Complex media were prepared in McIlvaine buffer (half-strength) (pH 4.0). Nutrient additions were made to bring the final concentration in the medium to 0.1%. Incubation was at 42°C.

TABLE 2. Effects of amino acids on germination[a]

Amino acid	% Germination after 8 h[b]
Proline	51
Alanine	51
Asparagine	48
Glutamine	42
Aspartic acid	40
Glutamic acid	34
Ornithine	23
Arginine	17
Tyrosine	6
Phenylalanine	5
Isoleucine	3
Leucine, glycine, histidine, lysine, methionine, serine, threonine, tryptophan, valine, hydroxy-proline, cysteine control	0

[a] Viability of the stock spore suspension was 65%; incubation was at 42°C; pH of the medium was 4.0.
[b] Experimental error, ±5%.

germinated within 6 h. Before the emergence of germ tubes, the sporangiospores swelled to an average diameter of 12.1 µm.

Sporangiospores neither swelled nor germinated during a 24-h incubation (42°C) in a carbon-deficient medium [i.e., buffer-$(NH_4)_2SO_4$]. However, after 24 h at 30°C in this medium, 7% of the spores germinated and produced chlamydospores. During germination, the sporangiospores swelled to 8.4 to 12.6 µm and subsequently gave rise to one to four chlamydospores per emerging hypha.

Germination kinetics in defined and complex media. The germination of sporangiospores was examined in several types of media, including those containing an enzymatic digest of casein (NZ amine type A), an acid digest of casein (Hy-Cas), or an enzymatic digest of soya (soy peptone). The kinetics of germination in these media are shown in Fig. 3. The enzymatic digests of soya and casein were more effective in promoting germination than either the acid digest of casein or the defined medium. The medium containing the acid digest of casein was notably poor as a germination medium.

To test the possibility that nutrients other than amino acids were responsible for the more rapid germination observed in NZ amine type A, a formulation of amino acids was prepared to duplicate the amino acid content of casein. Media were prepared in McIlvaine buffer (half-strength), pH 4. Nutrients were added to yield a final concentration of 0.1%. Incubation was at 4°C. Although the onset of germination occurred 30 min earlier in the NZ amine type A medium, the overall germination kinetics in the two media were quite similar. It is likely that promotion of germination by NZ amine type A is largely attributable to its amino acid content.

Effects of individual amino acids on germination. For an assessment of the relative effectiveness of individual amino acids on germination, a solid medium containing McIlvaine buffer (half-strength) (pH 4.0), 0.37% glucose, and 1.2% Ionagar was prepared. The addition of individual L-amino acids (analytical grade) was made aseptically to adjust the final concentration in the medium to 0.01 M. The results of these experiments are summarized in Table 2. Of the amino acids tested, L-proline and L-alanine were most effective in promoting germination. Leucine, cysteine, glycine, histidine, lysine, methionine, serine, threonine, tryptophan, va-

line, and hydroxyproline did not support germination after 8 h of incubation.

Germination in the presence of 10^{-6} M L-proline was 12%. Germination in the presence of 10^{-6} M L-alanine was 5%. A 10^{-5} M concentration of L-asparagine allowed 4% germination; however, L-glutamine did not promote germination even at 10^{-4} M. For comparison, germination in the presence of NZ amine type A occurred at 0.001%, a concentration that provided a calculated proline concentration of 10^{-5} to 10^{-6} M. Germination did not occur at concentrations of ammonium sulfate below 10^{-4} M.

Amino acid combinations were also tested for detection of any additive or synergistic effects. The results of these experiments are summarized in Table 3. Proline-alanine and proline-aspartic acid were the most effective combinations tested. These combinations, however, were only slightly more effective than proline or alanine alone. The striking inhibition of proline-induced germination in the presence of arginine, ornithine, or glutamine remains unexplained.

Effects of carbohydrates on germination of sporangiospores. The ability of various carbohydrates to promote sporangiospore germination was investigated by using a solid medium containing McIlvaine buffer (half-strength) (pH 4.0), 1.2% Ionagar, 0.05% $(NH_4)_2SO_4$, and 0.37% carbohydrate (reagent grade). Incubation was at 42°C for 8 h. The results of these experiments are summarized in Table 4. Of the various carbohydrates, glucose, mannose, xylose, fructose, soluble starch, and galactose were superior in promoting germination (greater than 40%). Germination in sorbitol, maltose, sucrose, mannitol, and trehalose ranged from 38 to 17%. Raffinose, ribose, lactose, and arabinose were less effective in promoting germination (less than 10%).

Germination reserves in sporangiospores. The ability of *R. oligosporus* to absorb sufficient nutrients during phase I for germination to occur after transfer to a non-nutritive medium was examined. For these experiments, spores were first incubated for 2 h in a nutrient-rich medium containing 0.1% NZ amine type A and 0.225% glucose in McIlvaine buffer (half-strength) (pH 4.0). After incubation (42°C, 150 rpm), spores were recovered by centrifugation (1,000 × *g*; 10 min). The supernatant was discarded, and residual medium was removed from the inner surface of the tube with a sterile cotton swab. The pellet was suspended in sterile physiological saline solution containing Tween 80. Portions of this suspension were then spread over the surface of plates containing McIlvaine buffer solidified with 1.2% Ionagar. After transfer to the non-nutritive medium, spores were evaluated for both swelling and germination. A control suspension of sporangiospores (not incubated in germination medium) was directly spread over the surface of the non-nutritive medium. These spores retained their original diameter (5.1 μm) after 5 h of incubation. Spores incubated in the germination medium for 2 h before transfer had swelled to 8.4 μm; however, both swelling and germination were arrested when the sporangiospores were transferred to the non-nutritive medium. It appears that effective pools of germination nutrients are not formed in *R. oligosporus* during phase I. Instead, continuous uptake of exogenous nutrients is required for subsequent germination.

TABLE 3. Effects of amino acid combinations on germination[a]

Combination	% Germination after 8 h[b]
Proline-alanine	61
Proline-aspartic acid	54
Proline-asparagine	50
Alanine-glutamic acid	50
Alanine-aspartic acid	49
Proline-glutamine	44
Alanine-glutamine	44
Asparagine-aspartic acid	40
Asparagine-glutamine	36
Asparagine-glutamic acid	36
Asparagine-arginine	36
Glutamine	36
Alanine-ornithine	36
Glutamic acid-ornithine	33
Asparagine-ornithine	32
Glutamic acid-arginine	31
Aspartic acid-glutamic acid	30
Aspartic acid-ornithine	29
Aspartic acid-arginine	29
Glutamine-glutamic acid	28
Glutamine-ornithine	24
Ornithine-arginine	20
Proline-glutamic acid	8
Proline-ornithine	8
Proline-arginine	8

[a] Viability of the stock spore suspension was 65%; incubation was at 42°C; pH of the medium was 4.0.
[b] Experimental error, ±5%.

TABLE 4. Effects of carbohydrates on germination[a]

Carbohydrate	% Germination after 8 h[b]
D-Glucose	59
D-Mannose	50
D-Xylose	50
D-Fructose	46
Soluble starch	44
D-Galactose	42
D-Sorbitol	38
Maltose	27
Sucrose	20
D-Mannitol	19
Trehalose	17
Arabinose	8
D-Ribose	7
Raffinose	6
Lactose	4
Dulcitol	0
Control	0

[a] Viability of the stock spore suspension was 65%; incubation was at 42°C; pH of the medium was 4.0.
[b] Experimental error, ±5%.

DISCUSSION

The results of these studies may have practical implications in the use of sporangiospore starters for tempeh fermentation. The fermentation is most commonly performed at 30 to 32°C. The most rapid germination of *R. oligosporus* sporangiospores, however, occurs at ca. 42°C. Therefore, the inclusion of a short initial incubation at 42°C before the product is transferred to 30 to 32°C may expedite the critical, initial portion of the fermentation and decrease the total fermentation time. Steinkraus et al. (7) have recommended an initial acidification of the substrate, to minimize the growth of bacterial contaminants during the fermentation. The results of the present study confirm that acidification of the substrate to ca. pH 4.0 favors rapid germination of

sporangiospore starters to establish competitive mycelial growth.

The morphological changes that occur during the germination of sporangiospores among the mucorales have been well documented. The present study confirms that germination of *R. oligosporus* sporangiospores occurs in two distinct phases. Neither swelling nor germ tube extension occurs when these sporangiospores are suspended in distilled water or buffer. Swelling (phase I) requires the presence of a suitable carbon source; germ tube extension (phase II) requires the simultaneous presence of carbon and nitrogen sources. When sporangiospores are incubated in the presence of exogenous nutrients and then transferred to a non-nutritive medium, swelling and germ tube extension are arrested. Therefore, it may be concluded that these sporangiospores do not contain appreciable endogenous reserves of nutrients. Ekundayo and Carlile (1) have reported that, in the presence of glucose, *Rhizopus arrhizus* sporangiospores can absorb sufficient nutrients within the first 2 h of incubation to complete germination in the absence of exogenous nutrients several hours later. The experimental method used by these authors, however, may have allowed nutrient carry-over to occur. In the present study, precautions were taken to minimize this possibility.

The most rapid germination of *R. oligosporus* sporangiospores occurred in mixtures of amino acids. L-Proline and L-alanine were particularly effective in promoting germination. It is interesting that when L-proline was used in combination with equimolar concentrations of L-glutamic acid, L-ornithine, or L-arginine, the effectiveness on germination was diminished. This effect remains unexplained. The ability of L-proline and L-alanine to promote germination at concentrations as low as 10^{-6} M suggests that their stimulatory effect is n t directly related to their utilization as carbon or nitr gen sources. Other active compounds containing a molar equivalent of nitrogen were not capable of supporting germination at these concentrations. Therefore, these amino acids may have a catalytic or regulatory role in promoting germination. Weber (8) and Weber and Ogawa (9) found L-proline to be the most effective of 60 compounds tested for stimulating the germination of *R. arrhizus* and *Rhizopus stolonifer* sporangiospores. These authors also found only small amounts of L-proline in the endogenous amino acid pool. Proline-stimulated germination was reported to require the presence of phosphate in the medium. Our findings indicate that proline-stimulated germination in *R. oligosporus* sporangiospores is optimized by the simultaneous presence of glucose and phosphate in the medium. Further investigations are required to determine the specific role of amino acids in influencing sporangiospore germination.

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